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Antibody Response to Crude Cell Lysate of *Propionibacterium acnes* and Induction of Pro-Inflammatory Cytokines in Patients with Acne and Normal Healthy Subjects

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***Propionibacterium acnes* (P. acnes) plays an important role in the disease pathogenesis of acne vulgaris, a disorder of pilosebaceous follicles, seen primarily in the adolescent age group. In the present study, the presence of antibodies against *P. acnes* (MTCC1951) were detected in acne patient (n=50) and disease free controls (n=25) using dot-ELISA and Western blot assay. The ability of *P. acnes* to induce pro-inflammatory cytokines by human peripheral blood mononuclear cells (PBMCs), obtained from acne patients and healthy subjects, were also analysed. The patients (n=26) who were culture positive for skin swab culture, were found to have a more advanced disease and higher antibody titres (1:4000 to >1:16000) compared to the *P. acnes* negative patients (n=24) and normal controls (n=25). An analysis of patients' sera by western blot assay recognized a number of antigenic components of *P. acnes*, ranging from 29 to 205 kDa. The major reactive component was an approximately 96 kDa polypeptide, which was recognised in 92% (24 of 26) of the patients sera. Further, the *P. acnes* culture supernatant, crude cell lysate and heat killed *P. acnes* whole cells, obtained from 72-h incubation culture, were observed to be able to induce significant amounts of IL-8 and tumor necrosis factor alpha (TNF-α) by the PBMCs in both the healthy subjects and patients, as analysed by cytokine-ELISA. The levels of cytokines were significantly higher in the patients than the healthy subjects. A major 96 kDa polypeptide reactant was eluted from the gel and was found to cause dose dependent stimulation of the productions of IL-8 and TNF-α. Thus, the above results suggest that both humoral and pro-inflammatory responses play major roles in the pathogenesis of acne.**

***Key words*: *Propionibacterium acnes*, acne rulgaris**

Acne vulgaris is a chronic inflammatory disorder of pilosebaceous folli cles, seen primarily in the adolescent age group (Cunliffe, 1989; Plewig and Kligman, 1993). Most patients have a mixture of non-inflammatory (comedones) and inflammatory (papules, pustules, and nodules) lesions. According to the recent report of White (White, 1998), nearly 40-50 million people in the USA have acne. However, data from the Indian population are scanty (Pandey, 1983). The universality of acne has resulted in significant efforts to treat this disease, but the precise mechanisms that govern the development and progression of acne remain to be understood.

Propionibacterium acnes plays an important role in the pathogenesis of acne vulgaris (Unna, 1896; Flemming, 1909; Strauss and Kligman, 1960). Although, *P. acnes* is a part of normal skin flora, it is evident that subjects with inflammatory acne have markedly elevated anti-propionibacterial antibody titres (Ingham *et al.*, 1987). Flemming (1909) demonstrated an increase in the opsoni-zation of acne bacillus with acne patient serum. This was further strengthened by the correlation demonstrated between the severity of acne and titre of antibody to *P. acnes* in patients' sera (Puhvel *et al.*, 1984; Puhvel *et al.*, 1966). *P. acnes* is a potent activator of a complement system by both classical and alternative pathways. Complement activation results in the production of C5a, which in turn causes the generation of neutrophil chemotactic factors (Webster *et al.*, 1978; Webster *et al.*, 1981; Webster and McArthur, 1982). Comedones itself can be caused by this activated complement. Serum containing a high anti-*P. acnes* antibody titre enhances this activation and preabsorption of the serum, and *P. acnes* ablates the enhancement (Webster *et al.*, 1979). *P. acnes* also stimulates exter-nalization of neutrophil lysosomal hydrolytic enzymes (Webster *et al.*, 1980). Pro-inflammatory cytokines, such as interleukin-1α (IL-1α), IL-1β, IL-8 and TNF-α (Vowels *et al.*, 1995), are implicated in the pathogenesis of acne. The interaction of *P. acnes* with the inflammatory system is magnified when an antibody to the organism is present, suggesting a potentially important role for anti-*P. acnes* antibody and proinflammatory cytokines in inflammatory acne. These cytokines have been shown to result in the expression of

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vascular and dermal adhesion molecules (Frank *et al.*, 1995), the chemoattraction of inflammatory cells (Kozlowska *et al.*, 1998) and the stimulation of other inflammatory mediators, such as the leukotrienes and prostaglandins (Helgren *et al.*, 1979).

In the present study, the prevalence of *P. acnes* in acne patients in the Indian population was investigated and the immunodominant antigen of *P. acnes* sought. Sero-reactive proteins of *P. acnes* were identified to define the antigenic substance(s). The ability of crude cell lysate of *P. acnes* (CCL), heat killed *P. acnes* (PA), culture supernatant of *P. acnes* (CS) and the eluted 92 kDa antigen (EA) for the direct induction of the expressions of pro-inflammatory cytokines from monocytes were also examined.

Materials and Methods

Materials

The lipopolysaccharide (LPS; *Escherichia. coli* 055:B), RPMI-1640, Histopaque, acrylamide, bisacrylamide, β-mercaptoethanol, *N,N,N,N*-tetramethylethylene diamine (TEMED), phenylmetanesulfonylfluoride (PMSF), aprotinin, leupeptin were obtained from Sigma Chemicals (USA). The TNF-α and IL-8 ELISA kits were obtained from R&D system (USA). The FCS, dithiothreitol (DTT) and antibiotic mix were obtained from Life Technologies (USA), the protein molecular weight marker and goat anti-human IgG-HRP conjugate from Bangalore Genei (India) and the Tris base, glycine, sodium dodecyl sulphate, bovine serum albumin, sucrose and Tween-20 from SRL (India). The nitro-cellulose paper (NCP, 0.45 μ) was obtained from Millipore (USA) and the Muller Hinton and Brucell HK agar medium from BBL (Tokyo, Japan).

Subjects

Fifty patients (29 males and 21 females), with a mean age of 20.4 ± 5, ranging from 13 to 35 years, with inflammatory acne vulgaris were selected for the investigations. All the patients had acne vulgaris in stages I, II, III, or IV, with at least one-year-old history. Patients with the mildest disease having only few dry comedones fell into grade-I, those with large number of papules, comedones and few pustules fell into grade-II. Patients with a grade-III disease had large numbers of pustules, in addition to discharging papules and comedones. Patients in grade IV had abscesses besides all of the above clinical features. None had been treated with systemic antibiotics for at least 3 months prior to the start of the investigation. Twenty-five normal healthy individuals (15 males and 10 females), with a mean age of 23.6±4.1, ranging 15 to 30 years, without acne vulgaris were included as healthy control subjects.

Identification of strains

Skin swab cultures and biochemical assays were performed on both the patients' and healthy subjects' samples. After cleansing acne lesions with 70% ethanol, the contents were squeezed out and collected in RCM (Robertsons cooked meat) broth tubes (Whiteside and Voss, 1973). The contents were inoculated onto agar medium supplemented with 5% defibrinated horse blood, and incubated both aerobically and anaerobically for 72 h. Whitish convex shaped colonies were picked up and Gram staining was performed. The Gram-positive, non-spore forming, rods were preliminarily labeled as *P. acnes* and were further characterized by biochemical tests (Moore and Vholdeman1974). The sera of the healthy subjects were collected and stored at -20°C until tested.

Preparation of P. acnes *antigens, crude cell lysate (CCL), culture supernatant (CS) and heat killed* P. acnes *(PA)*

The *P. acnes* bacteria, MTCC No. 1951, was procured from IMTECH, Chandigarh, India and was cultured anaerobically on blood agar, containing 0.1% Tween 80, for 5 days. A suspension in phosphate buffered saline (PBS), corresponding to a 2.0 McFarland standard, was prepared from this culture. Two milliliters of this suspension was transferred aseptically to a 500-ml culture flask containing 300 ml of freshly prepared 1% peptone, 0.5% yeast extract and 0.1% glucose (PYG) medium, supplemented with 0.004 M magnesium, manganese, iron salts, 0.01% cysteine and 0.1% Tween 80. Flask was incubated for 72 h at 37°C in anaerobic atmosphere (N_2-80%, CO_2-10%, H_2-10%). The log phase bacterial culture was centrifuged at 5000×g and 4°C for 20 min and the culture supernatant (CS) removed, filtered (through a 0.22-μm-pore size filter) and stored at -20°C. The bacterial pellet was washed three times in chilled 100 ml PBS and finally suspended in 10 ml of the same. The crude cell lysate (CCL) was prepared by sonicating 6 ml of bacterial suspension, on ice, for 5 pulses of 30 sec each using sonicator (Heat system ultrasonic Inc., USA), which was then supplemented with protease inhibitors (PMSF 1mM; DTT 1mM; Aprotinin 2 μg/ml; & Leupeptin 2 μg/ml). The disrupted organisms were centrifuged at 15,000× g and -20°C for 1h, and the protein concentration of the supernatant estimated according to the Lowry methods (Lowry *et al.*, 1951) and then stored at 20°C. A further 4 ml of the *P. acnes* suspension was incubated at 80°C for 30 min to heat kill the bacteria. The heat killed *P. acnes* (PA) suspension was stored at 4°C until used.

Dot-ELISA

A 2 μl/spot of the crude cell lysate of *P. acnes* was spotted on nitrocellulose paper (NCP, Bio-Rad, USA) and air-dried for 30 min. The NCP was blocked with 1% bovine serum albumin (BSA) in tween-20 tris buffered saline (T/TBS, 0.05% Tween-20, 10 mM Tris and 150 mM NaCl (pH 7.5)) for 1h, and subsequently washed three times with T/TBS. The NCP was then kept on wet filter paper

in a moist chamber, and undiluted sera (2 µl/spot) were added to the antigen spots. After 1h of incubation at 37°C the NCP was washed and incubated with anti-human IgG horseradish peroxidase conjugate (1 : 500) for 1 h at 37°C. Finally, the NCP was developed using 3-3 diaminobenzidine (DAB, 6 mg/10 ml with 10 µl of H_2O_2) as a chromogenic substrate. The development of well-defined dark brown spots was considered a positive reaction.

Electrophoresis, immunoblotting, and elution technique

The crude cell lysate of *P. acnes* (25 µg) was separated by 10% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970), with minor modifications. A mixture of standard protein markers (MW 29 kDa to 205 kDa) was used for the determination of the molecular weight. A total of 20 µg of protein per lane was loaded onto the gel and was subjected to electrophoresis. The resolved polypeptide bands were either detected by staining with coomassie brilliant blue stain or electrophoretically transferred to NCP for immunoblotting. Immunoblotting was carried out as described by Towbin *et al.* (1979). After transfer, the membrane was blocked overnight in blocking solution (3% BSA in tris-buffered saline, pH 7.5) and cut into vertical strips. Subsequently, the strips were washed with T/TBS (0.05% Tween 20 in TBS) for 10 min, and then incubated with patients sera (1 : 200 dilution) for 2 h at 37°C with gentle rocking, followed by washing three times with T/TBS. After being washed, the strips were incubated at 37°C for 1½ h with detection antibody, horseradish peroxidase-conjugated goat antibody to human-IgG (1 : 500). The color was developed using DAB (6 mg/ml with 10 µl of H_2O_2) and the reaction stopped by the addition of distilled water.

Preparation of eluted antigen (EA)

The extraction of protein from preparative acrylamide gels was accomplished by the passive protein diffusion methods (Bhown and Bennett, 1983). The specific band of 96 kDa was cut into small pieces and homogenized in 0.01 M NH_4HCO_3 and 0.05% SDS buffer, and kept at 37°C overnight. After incubation, the suspension was centrifuged at 15,000x g and -20°C for 30 min. The eluted antigen (EA)/supernatant was pooled, and the protein concentration estimated by the Bradford assay (1976). A small quantity of sample was again run on SDS-PAGE for confirmation of the protein band.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were separated from the venous blood of the healthy volunteers and patients with acne by density gradient centrifugation (Boyum, 1968). The blood was diluted 1:2 with phosphate-buffered saline (PBS) (pH~7.2), layered on Histopaque, washed thrice with PBS and resuspended in complete RPMI-1640 supplemented with 10% heat inactivated FCS.

To determine the effect of heat killed *P. acnes* (PA) on human PBMCs, the *P. acnes* culture supernatant (CS), crude cell lysate (CCL) and eluted antigen (EA) of eight subjects with moderate to severe acne, and six healthy subjects with no clinical evidence of acne, were included in the study. The PBMCs (10^6/ ml) were incubated with increasing concentrations of PA, CS, CCL and EA and with 10ng/ml lipopolysaccharide (LPS). Cultures without stimulants were used as controls. All cultures were incubated at 37°C in a 5% CO_2 and 95% humidity atmosphere for 18 h; supernatants were collected by centrifugation (10 minutes at 3000×g), and stored at -70°C. The viability of the cells at the end of the culture, as estimated by the trypan blue exclusion test, was >85% in all cases. Cell free supernatants were harvested and assessed for IL-8 and TNF-α by an enzyme-linked immunosorbent assay (ELISA).

Quantification of cytokines

Cell free supernatants were analysed for IL-8 and TNF-α using commercially available ELISAs. The sensitivities of the IL-8 and TNF-α assays were 32 and 15 pg/ml, respectively.

Statistical analysis

The differences in the cytokine production by various stimulatory agents were analysed using the Wilcoxon signed rank test for paired data and those between the patients and controls by the Mann-Whitney U test. A p value <0.05 was considered to be statistically significant.

Results

Skin swab culture analysis

Skin swabs from 50 patients with acne vulgaris and 25 healthy subjects were cultured for identification of *P. acnes*. Twenty six out of the 50 patients were positive for *P. acnes,* where 4 patients were grade IV, 13 grade III and 9 grade II. Among the negative *P. acnes* patients, 16 were listed as grade II and 8 as grade I. Only 3 out of the 25 healthy subjects were positive for *P. acnes*.

Dot-ELISA

The analyses of the anti-propionibacterial antibodies, according to the clinical grade and *P. acnes* culture positivity, are shown in Table 1. Most of the *P. acnes* positive patient had antibody titres greater than 1 : 8000, while most of *P. acnes* negative patient had antibody titres less than 1 : 4000. Patients with the more severe grades of disease had higher antibody titres than those of less severity. None of the controls had titres more than 1 : 4000 (Table 1).

Table 1. Anti *P. acnes* antibody titres for the sera of normal healthy subjects, *P. acnes* culture positive and negative patients, by Dot-ELISA. Results are analysed according to the clinical grading of the disease

Antibody titre	*P. acnes* +ve patients, n=26				*P. acnes* –ve patients, n=24				Normal control, n=25
Grades (Number of patients)	I(0)	II(9)	III (13)	IV(4)	I(4)	II(20)	III(0)	IV(0)	
Titre									
nil	-	-	-	-	-	-	-	-	15 (60.0%)
1:400	-	-	-	-	-	-	-	-	-
1:500	-	-	-	-	4 (100%)	5 (25.0%)	-	-	-
1:1000	-	-	-	-	-	7 (35.0%)	-	-	6 (24.0%)
1:2000	-	-	-	-	-	3 (15.0%)	-	-	2 (8.0%)
1:4000	-	4 (44.4%)	-	-	-	2 (10.0%)	-	-	2 (8.0%)
1:8000	-	5 (55.6%)	5 (38.3%)	-	-	3 (15.0%)	-	-	-
1:16000	-	-	3 (23.0%)	-	-	-	-	-	-
>1:16000	-	-	5 (38.3%)	4 (100%)	-	-	-	-	-

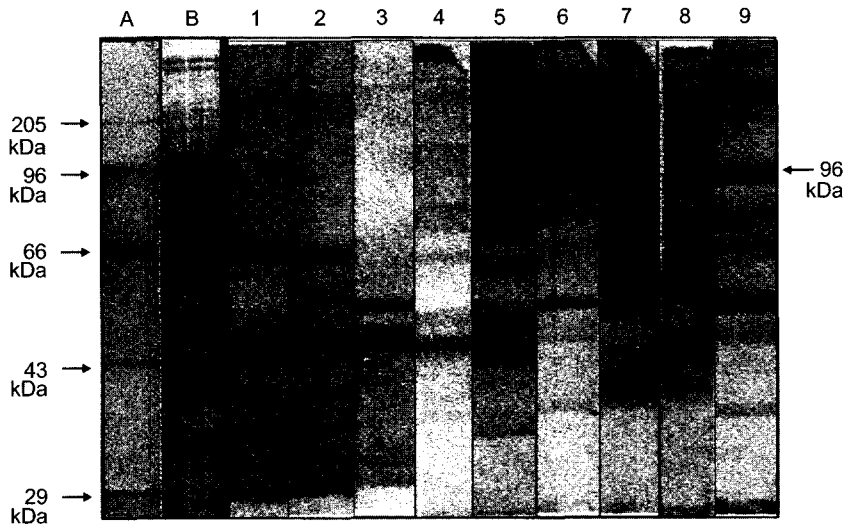

Fig. 1. SDS-PAGE (10% polyacrylamide) analysis of *P. acnes* crude cell lysate (CCL), and protein immunoblots of *P. acnes* crude cell lysate (CCL) separated by SDS-PAGE and reacted with patients' and healthy controls' sera. Lane A, molecular weight markers in kilodalton (kDa); Lane B, *P. acnes* crude cell lysate (20 μg). A Coomassie Brilliant-blue stain was used. Lanes 1-4 showed the cross reactivity with healthy control sera and lanes 5-9 the cross reactivity with patients' sera.

SDS-PAGE and western blot analysis of the* P. acnes *CCL

In the SDS-PAGE analysis and Coomassie Brilliant blue staining, *P. acnes* CCL revealed ~25 bands, with molecular weight ranging from 29 to 205 kDa (Fig. 1). Immunoblotting of these antigens on NCP, and their reaction with patients' sera, detected 12 major antigenic bands: 30, 43, 56, 60, 65, 72, 78, 85, 96, 130, 150 and 200 kDa (Fig. 1). One prominent antigenic band, 96 kDa in size, was found to react consistently with 24 of the 26 *P. acnes* positive patients' sera (92%). This band was not recognised in any of the control sera (Table 2).

Effect of* P. acnes *and its derivatives on inflammatory cytokine production:

i) Heat killed *P. acnes* (PA) stimulates IL-8 and TNF-α production.

Incubation of various concentrations of PA, with the PBMCs of both the healthy subjects and patients, resulted in a dose dependent production of pro-inflammatory cytokines. All PBMCs were observed to spontaneously release IL-8 and TNF-α into the cultures. The median spontaneous IL-8 production from the patients and healthy subjects were 3120 (range 1950-3750) and 3800 pg/ml (range 2400-5000), respectively. The IL-8 was produced in a greater abundance than the TNF-α. The IL-8 production from the healthy subjects and patients were 11050 and 8360 pg/ml, respectively, when incubated with 10^9 *P. acnes* organisms per ml (Fig. 3A and 3B). A decreased, yet significant, dose dependent production of IL-8 was still observed when the concentration of *P. acnes* was reduced 10 or 100-fold.

The PBMCs were also shown to produce large quantities of TNF-α (Fig. 2A and 2B) in a dose dependent response similar to that seen for IL-8. The median spon-

Table 2. Percentage recognition of each antigenic component of *P. acnes* antigens in the sera of *P. acnes* positive, *P. acnes* negative and healthy controls, as demonstrated by immunoblotting

Antigen (kDa)	% Recognition		
	Patients		Controls
	P. acnes positive	*P. acnes* negative	Healthy controls
200*	74	25	-
150*	46	48	-
130*	38	32	-
96*	92	28	-
85*	46	42	-
78*	78	30	-
72*	46	50	-
65*	69	62	-
60*	57	52	24
56*	50	42	-
54	8	-	-
52	15	12	-
50	38	32	-
45	8	12	8
43*	69	60	48
41	42	40	40
38	8	-	24
36	30	60	40
34	38	12	32
32	23	6	-
30*	73	30	12
28	15	45	28
27	8	-	16
26	15	-	-
25	4	-	-

taneous TNF-α production from the patients and healthy subjects were 720 (range 200-1200) and 320 pg/ml (range 120-480 pg/ml), respectively. Significant differences in cytokine production were observed between the patients and healthy subjects

ii) *P. acnes* culture supernatant (CS) stimulates IL-8 and TNF-α production.

P. acnes CS, grown in PYG medium for 72 h, was collected and incubated with PBMCs at various dilutions, to determine if *P. acnes* may secret a factor capable of directly stimulating the PBMCs to produce pro-inflammatory cytokines. Analysis of cell CSs indicated a significant production of IL-8 in comparison with that produced by cells treated with PYG medium alone. The level of cytokine production was not as high as that observed with cells incubated with heat killed *P. acnes*; however, the amounts of the various cytokines produced were proportionally similar, *i.e.,* IL-8 production was higher than TNF-α. Significant differences in the cytokine production

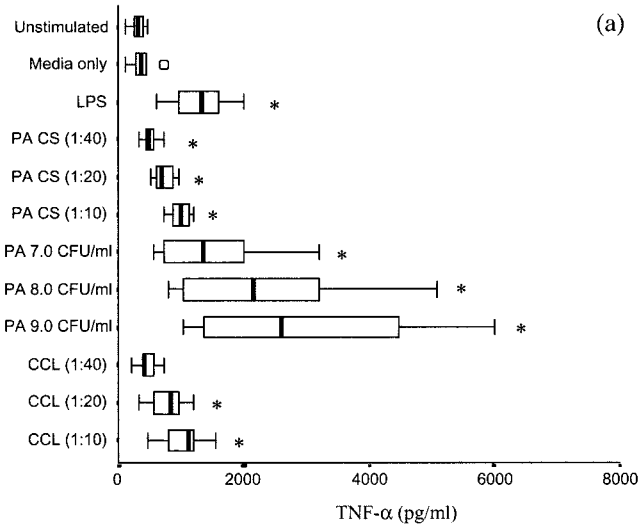

Fig. 2a. Effect of *P. acnes* antigens on the production of TNF-α by the PBMCs of the normal healthy controls. (*$p<0.05$ by "Wilcoxon sign rank test" as compared to the unstimulated control, CCL=crude cell lysate. PACS=culture supernatant, PA=heat killed *p. acnes*).

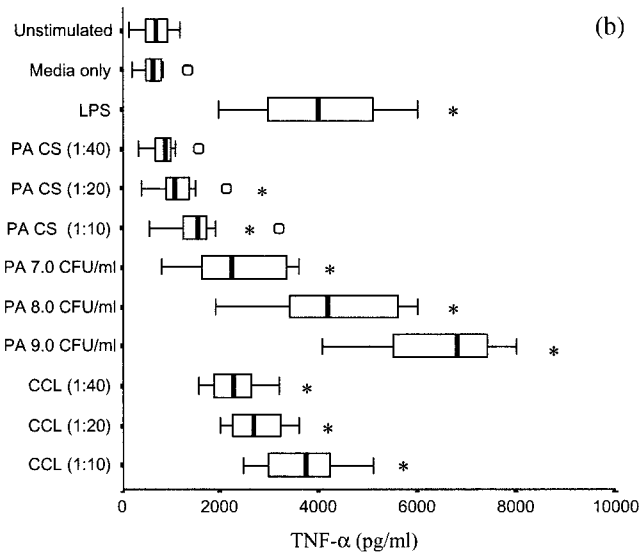

Fig. 2b. Effect of P acnes antigens on the production of TNF-α by the PBMCs of the acne patients. (*$p<0.05$ "Wilcoxon sign rank test" as compared to unstimulated control, CCL=crude cell lysate. PACS=culture supernatant, PA=heat killed *P. acnes*).

were observed between the patients and healthy subjects (Fig. 2A and 2B; 3A and 3B).

iii) *P. acnes* crude cell lysate (CCL) stimulates inflammatory cytokine production.

To study whether the cytosolic proteins of *P. acnes* have any stimulatory activity, PBMCs (patients and healthy subjects) were stimulated with crude cell lysate of *P. acnes*. It was observed that CCL, at 1:20 and 1:10 dilutions, significantly stimulated the levels of IL-8 and TNF-α; however, at a 1:40 dilution the CCL caused an increase in the IL-8 level only (Fig. 2a and 2b; Fig. 3a and 3b).

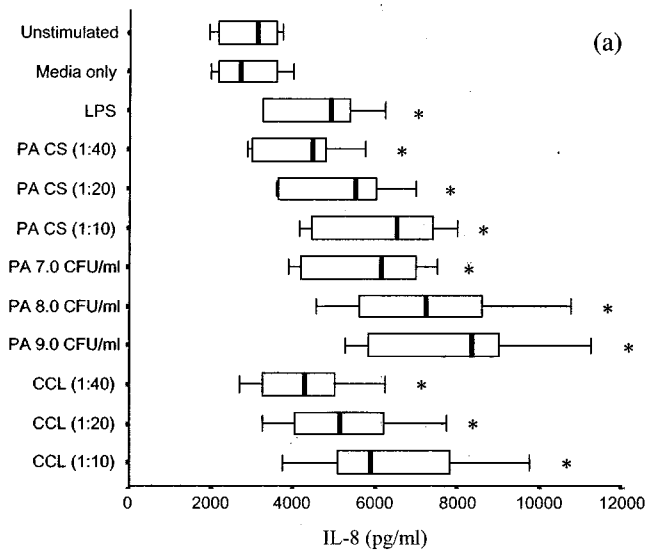

Fig. 3a. Effect of *P. acnes* antigens on the production of IL-8 by the PBMCs of the normal healthy controls (*$p<0.05$ "Wilcoxon sign rank test" as compared to unstimulated control, CCL=crude cell lysate. PACS=culture supernatant, PA=heat killed *P. acnes*).

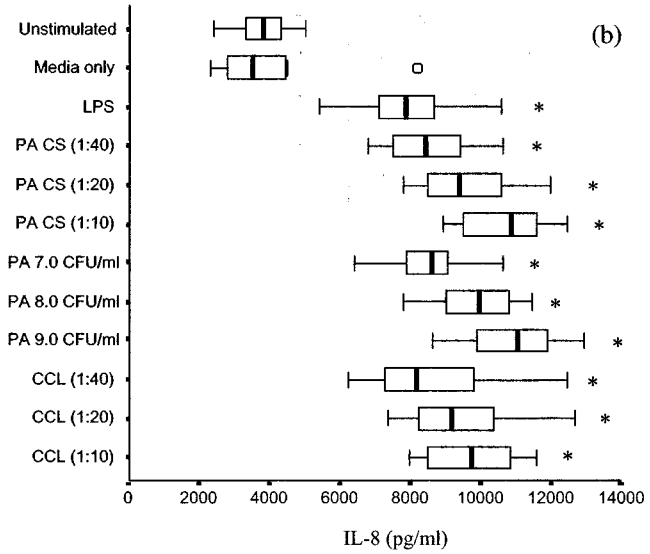

Fig. 3b. Effect of P acnes antigens on the production of IL-8 by the PBMCs of the acne patients (*$p<0.05$ "Wilcoxon sign rank test" as compared to unstimulated control, CCL=crude cell lysate. PACS=culture supernatant, PA=heat killed *P. acnes*).

Significant differences in the cytokine production were observed between the patients and healthy subjects.

iv) *P. acnes* eluted antigen (EA) stimulates inflammatory cytokine production.

In order to identify the component of *P. acnes* with the immuno-modulatory activity, PBMCs of healthy subjects and patients were stimulated with the most sero reactive eluted antigen (~96 kDa) of *P. acnes*. An analysis indicated significant productions of IL-8 and TNF-α compared with those produced by cell treated with PYG medium alone (Table 3). The level of cytokine production was not as high as that observed with cells incubated with heat killed, CS and CCL of *P. acnes;* however, the amounts of various cytokines produced were proportionally similar i.e., IL-8 production was higher than TNF-α. Significant differences in the cytokine production were observed between the patients and healthy subjects (Table 3).

Table 3. Effect of eluted antigen on the production of cytokines by the PBMCs of the normal healthy controls (n=6) and patients with acne vulgaris (n=8), as estimated by ELISA

	TNF-α (pg/ml) Median (range)		IL-8 (pg/ml) Median (range)	
	Normal (n=6)	Patients (n=8)	Normal (n=6)	Patients (n=8)
Unstimulated	320 (120-480)	720 (200-1200)	3120 (1950-3750)	3800 (2400-5000)
LPS	1340* (600-2000)	4000* (600-5440)	4930* (3240-6250)	7880* (5400-10600)
Eluted Ag (1μg/ml)	280 (200-720	560 (150-1360)	2850 (1800-4000)	4800* (3200-7200)
Eluted Ag (5μg/ml)	420* (240-1120)	1000* (480-1800)	3275* (2100-4250)	6500* (5120-7680)
Eluted Ag (15μg/ml)	500* (360-1560)	1580* (840-2400)	3460* (2275-4280)	7760* (6240-9420)

*$p<0.05$ as compared to unstimulated control by "Wilcoxon sign rank test"

Discussion

Of the three major organisms present on skin surface, namely *P. acnes*, *S. epidermidis* and *Malassezia furfur, P. acnes* is considered the most important in the pathogenesis of acne vulgaris (Leeming *et al.*, 1984; Leeming *et al.*, 1985; McGinley *et al.*, 1978). Although there have been reports from other parts of the world (Gehse *et al.*, 1983), our findings suggest that 52% of the patient with acne vulgaris carry *P. acnes* in their acne lesions. This is the first study on the prevalence of *P. acnes* in the Indian population. The carriage rate of *P. acnes* was considerably below that found by Leeming *et al.* (1988), who reported the presence of organisms in 79% of the patients in their study. The lower rate in our population may be due to the low prevalence of the organism in Indian patients.

Of the patients enrolled in the present study, the presence of antibodies against *P. acnes* (MTCC1951) was detected using Dot-ELISA and Western blotting techniques. Of 50 patients, 26 were positive for the presence of *P. acnes* in their lesions and had higher antibody titres compared to the *P. acnes* negative patients. Antibody titres in healthy subjects were absent in 80% of cases, while only 20% of cases showed elevated titres. The presence of high antibody titres in acne vulgaris patients compared to those in healthy subjects suggest that *P. acnes* has an immunogenic antigen that stimulates a strong humoral

immune response. Our results are supported by the study of Iversen *et al.* (1985), who showed the presence of antibodies directed against specific *P. acnes* antigens in acne patients.

Antibodies to *P. acnes* rise in proportion to the severity of an acne inflammation and in this study higher titres were observed in patients having disease severity of grades III and IV, whereas low titres were seen in the less severe disease (Table 1). Inflammatory acne is due to hypersensitivity to *P. acnes,* and evidence supports this theory. *In-vitro* studies have shown that complement activation by comedonal material is stimulated by the presence of antibodies to *P. acnes,* and is greatly decreased by their removal. Similarly, the organism itself is a much more potent activator of the complement when the anti-*P. acnes* antibody is present; and therefore, will generate more C5a and subsequently attract more neutrophil in the immune patients (Webster *et al.*, 1978). Ingham *et al.* (1987) made similar observations, and have shown that antibodies to whole *P. acnes* cells rise in proportion to the severity of acne. In addition, they found that the IgG subtype was predominant in severe acne, while IgM was the main immunoglobulin in the less severe disease, suggesting that severe acne is associated with a durable immune response. The antibody to *P. acnes* is also required to trigger the release of neutrophil lysosomal hydrolases (Webster *et al.*, 1980). Thus, all potential explanations confirm that, in presence of elevated antibody titres, *P. acnes* is a much more potent and damaging inflammatory stimulus.

Dot-ELISA provides limited information; therefore, western blotting was performed to detect the specific immunogen of *P. acnes* against which antibodies are directed. Analysis of patient sera for reactivity to *P. acnes* shows differences in the pattern of the antibody response. Patient sera recognised approximately 12 immunogens on blotting, which was in agreement with the earlier finding of Holland *et al.* (1993). Differences in the antibody pattern may reflect differences in the antigenic make up of the *P. acnes* harbored in the pilosebaceous follicles of different hosts. In our study, one major antigenic component of *P. acnes* was found, a 96-kDa-size polypeptide, which was recognised in 92% of the patients' sera and seems to play a major role in the disease pathogenesis.

To analyze the ability of *P. acnes* and its component to induce inflammatory responses in the disease, human PBMCs were stimulated with crude cell lysate of *P. acnes* and its derivatives (PA, CS and EA). These different derivatives of *P. acnes* were observed to directly stimulate the productions of the pro-inflammatory cytokines, IL-8 and TNF-α, by the PBMCs of both the patients and healthy subjects. The stimulations of *P. acnes* by PA and CS were specific, as the increases in the secreted levels of cytokines were dose dependent. The levels of stimulation seen with CCL and EA were less than those with PA and CS of *P. acnes*. Thus, the above findings suggest that the stimulation of pro-inflammatory cytokine is mainly caused by a component of the cell walls. Crude cell lysate and/or other antigens, if at all, play a very minor role in this stimulation. Our above finding is in agreement with the study of Vowel *et al.* (1995), who also showed that intact cells of *P. acnes,* and *P. acnes* CS, stimulated the productions of TNF-α, IL-1 and IL-8 in the human monocyte cell lines THP-1 and U-937, as well as in the human PBMCs of patients and healthy subjects.

It is suggested that tissue macrophages are the main source of these cytokines. *P. acnes,* which was classified previously as *Corynebacterium parvum* (Cummins and Johnson 1974), has been shown to process macrophages-activating activities, suggesting that it may be capable of eliciting the production of pro-inflammatory cytokines (Bomford and Christie 1975; Sljivic and Watson 1977; Ghaffar 1980). Vowels *et al.,* (1995) suggested that the peptidoglycon-polysaccharide (PG-PS) complex and lipoteichoic acid are the compounds that induce the production of pro-inflammatory cytokines. In addition to stimulation via the intact cells wall or cell wall component, the PG-PS complex can be secreted in a soluble form by gram-positive bacteria, thus inducing the monocytes to produce pro-inflammatory cytokines.

Thus, our finding, together with earlier reports, suggests that the stimulatory effects of *P. acnes* CS may be due to the presence of a soluble factor, possibly PG-PS. The soluble factor responsible for the production of pro-inflammatory cytokines can not be a heat sensitive enzyme, as heating of *P. acnes* to 80°C did not destroy its ability to induce cytokines from PBMCs. Vowels *et al.* (1995), using several pieces of evidence, has previously shown that PG-PS is responsible for the production of cytokines. They also found that the factor was approximately 3-30 kDa in size, which was consistent with soluble PG.

The spontaneous levels of IL-8 and TNF-α were observed to be higher in patients with acne compared to healthy subjects, suggesting that patients with severe acne have an overproduction of IL-8 and TNF-α. Earlier, Vowel *et al.* (1995) failed to observe such a correlation between the two groups, but attributed this to their inclusion of patients with less severe forms of the disease. The increase in cytokine levels in response to intact cells or CS of *P. acnes* was greater in the patient group than in the healthy subjects, suggesting a severe pro-inflammatory response in patients with acnes.

PG-PS has been previously shown to mediate several experimentally induced chronic inflammatory diseases in animal models. PG-PS generated in the sebaceous follicle interacts with tissue macrophages during migration through the intact follicular wall. This interaction takes place with the help of surface molecules, such as CD-14 and the toll like receptors present on monocytes/macrophages. This activates further signal transduction pathways, which ultimately end in the production of pro-inflammatory cytokines, like IL-1, IL-8 and TNF-α. These cytok-

ines further perpetuate the inflammatory response. The presence of an inflammatory disease only in the patients, and not in healthy subjects, may be due to the presence of a significantly higher density of *P. acnes* in patients with acne compared to healthy subjects.

Thus, in the present study on the northern Indian population, the prevalence for *P. acnes* was found in 52% of patients with acne vulgaris. In addition, elevated antibody titre levels were observed in patients, which directly correlated with the disease severity. We have identified an approximate 96 kDa putative immunogen present only in the patients with acne vulgaris. Finally, the ability to induce pro-inflammatory cytokine in patients with acne has been shown to be mainly due to a component of the cell walls, although there are also other factors that play very minor roles in the induction of cytokines from monocytes.

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